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Cucumber Seedling Indoleacetaldehyde Oxidase[1]

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ABSTRACT

Extracts of light-grown *Cucumis sativus* L. seedlings catalyzed the oxidation of indole-3-acetaldehyde to indole-3-acetic acid. No added cofactors were required. Inhibitor studies indicated that the enzyme is a metalloflavoprotein. While indole-3-aldehyde, benzaldehyde, and phenylacetaldehyde partially inhibited the oxidation of indole-3-acetaldehyde, suggesting that they may serve as alternative substrates, it is proposed that indoleacetaldehyde is the major substrate *in vivo*. 2,4-Dichlorophenoxyacetic acid strongly inhibited the indoleacetaldehyde oxidase activity, and it is proposed that this enzyme may be subject *in vivo* to feedback inhibition by indole-3-acetic acid. The enzyme was activated by brief heating or by treatment with mercaptoethanol.

The young cucumber shoot is an excellent system for studies of auxin biosynthesis and its regulation. The growth of the intact seedling is strongly promoted by exogenously supplied IAA or synthetic auxins (5), suggesting a regulatory mechanism which disallows saturating auxin synthesis under normal conditions. We have isolated and characterized several of the enzymes presumed to be responsible for auxin synthesis in cucumber (1, 2, 6, 12) and have shown that certain of them are subject to regulation (7; and H. M. Brown and W. K. Purves, manuscript in preparation). The present report extends these studies to include the characterization of the cucumber indole-3-acetaldehyde oxidase and its regulatory properties.

The derivation of IAA from IAAld[5] *in vivo* has been demonstrated by several workers, and it is now accepted that the enzymic oxidation of IAAld to IAA is the terminal step in auxin biogenesis (11). Rajagopal (9, 10) has described the properties of a partially purified aldehyde oxidase activity from *Avena*, and Wightman and Cohen (15) reported an NAD-dependent aldehyde dehydrogenase in mung bean. In those reports, IAAld was treated as the principal substrate for the enzymes. Our initial attempts to demonstrate an IAAld-oxidizing activity in extracts of cucumber were unsuccessful, apparently because of the lability of the enzyme; but we now report its occurrence and possible regulatory role in auxin biosynthesis in cucumber. It is, of course, impossible to state unequivocally that the enzyme described here serves to oxidize IAAld *in vivo*.

MATERIALS AND METHODS

Preparation of Enzyme. Seeds of *Cucumis sativus* L. cv. National Pickling (Burpee Seed Co.) were soaked for 2 hr in tap water and sown in vermiculite saturated with tap water. Seedlings grew for 7 days under a 14-hr light, 10-hr dark cycle at 25 C. Shoots were homogenized in ice cold 50 mM tris buffer (pH 7.3) containing 1 mM $MgCl_2$, for 90 sec in a Waring Blendor. The homogenate was filtered through cheesecloth (eight layers) and centrifuged for 20 min at 10,000*g*. The supernatant fluid was filtered through glass wool to remove the lipid pellicle. For some experiments, this filtrate served as the enzyme preparation, while others involved a heat-treated preparation obtained as follows.

Aliquots (10 ml) of the filtrate were immersed in a 60 C circulating water bath and, at selected times, plunged into an ice bath. Except for the time study, the standard heating time was 2.5 min. The heated and chilled samples were centrifuged for 15 min at 10,000*g*. The supernatant fluid was decanted and used as "heat-activated" enzyme.

Some untreated or heat-activated preparations were also subjected to a pH precipitation procedure. The pH was slowly lowered to 5 with 1 N HCl at 0 to 4 C with constant stirring. The preparation was centrifuged at 10,000*g* for 15 min. The pH of the supernatant fluid was raised to 6.2 with 1 N NaOH and this solution was employed as enzyme without further purification.

Enzyme Assay. The product of aldehyde oxidase action upon IAAld is IAA. The assay was based on the colorimetric determination of this product with Salkowski reagent (3). A typical reaction mixture consisted of 0.2 ml of 0.8 mM IAAld (in water), 0.1 ml of water or of a test compound, and 0.1 ml of enzyme preparation. The reaction was stopped with 0.1 ml of 10% (v/v) trichloroacetic acid, and the resulting precipitate was removed by low speed centrifugation. Salkowski reagent (0.5 ml) was added to the supernatant fluid, and the *A* at 529 nm was taken against a water blank with a Beckman Acta V spectrophotometer following a 20-min incubation period to allow color development. The reaction times varied (from 3 to 30 min) depending upon the activity of the enzyme preparation and the nature of the particular experiment, but most reported rates were based on 3- or 10-min runs. Several time points were obtained to insure that the reaction rate was constant over the time period employed. The reaction velocity was taken as the slope of plots of A_{529} *versus* time.

Preparation of Reagents. IAAld bisulfite (Sigma Chemical Co.) was stored desiccated at −20 C, and free IAAld was prepared before each experiment. An aqueous solution of IAAld bisulfite was made, the pH raised to 10, and the precipitated free IAAld extracted with diethyl ether. After the addition of water, the ether was removed in a flash evaporator. The final concentration of free IAAld in water was determined from the A_{280} by application of Beer's law with ϵ_M^{280} = 5,400 liter·mol^{-1}·cm^{-1} (2).

Salkowski reagent was prepared by the method of Gordon and Weber (3).

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[5] Abbreviations: IAAld: indole-3-acetaldehyde; IEt: indole-3-ethanol.

RESULTS AND DISCUSSION

Isolation of Enzyme Activity. Unheated extracts of green cucumber shoots catalyzed the oxidation of IAAld to IAA. Identification of the product was based upon the appearance in these reaction mixtures of Salkowski-positive material at the R_F of IAA on thin layer chromatograms in three solvent systems (Table I). No indole-3-ethanol was formed in these reaction mixtures. The presence of IAAld reductase activity in the extract, causing the formation of IEt (2), would have invalidated our assay system, since mixtures of IAAld and IEt produce an intense pink color with the Salkowski reagent (12).

The enzymic oxidation of IAAld to IAA was not enhanced by the addition of NAD^+, $NADP^+$, NADH, or NADPH. Studies of the substrate specificity and kinetic parameters of this enzyme must await the purification of the activity. Attempts at purification are hampered by the extreme lability of this enzyme. In both untreated and heat-activated (see below) preparations, 90% or more of the IAAld oxidase activity was lost at 4 C within 24 hr after the plants were homogenized.

Activation by Heat and Mercaptoethanol. The IAAld oxidase activities of both crude extracts and supernatants from acid precipitation were increased 2- to 10-fold by a mild heat treatment. Figure 1 (top) shows this activation as a function of preincubation time in a 60 C water bath. The response is exceedingly sharp, with marked inhibition of activity resulting from overexposure to heating. Strongest activation occurred with a 2- to 3-min heating period, while treatments of 7 min or longer reduced enzyme activity below that of the unheated control. Figure 1 (bottom) shows the activation response to a 2.5-min preincubation at several temperatures. Again, the enzyme showed activation and subsequent inhibition as the proper combination of temperature and incubation time was approached and passed. Heat-activated preparations were employed in the remaining experiments unless otherwise indicated.

Similar activation of the IAAld oxidase activity was observed upon treatment with mercaptoethanol. Homogenization of seedling tissue with tris buffer containing 1 mM mercaptoethanol, or addition of mercaptoethanol (final concentration, 1 mM) to crude homogenates, led to a comparable activation of this enzyme. We have also observed apparent activation of the IAAld oxidase by freezing of the crude homogenate.

Effects of pH and Chemical Inhibitors. The response of cucumber IAAld oxidase to pH is shown in Figure 2. In this experiment, the pH of the crude homogenate was lowered to 5, the resulting precipitate was removed by centrifugation, and the pH of the supernatant fluid was readjusted to the pH values shown. It can be seen that the IAAld oxidase has a rather stringent pH requirement centered near pH 6.2. It was shown that inhibition by pH values above and below the optimum was reversible, since readjustment of enzyme preparations at inhibitory pH values to pH 6.2 restored their activity. Subsequent experiments described in this paper were carried out in unbuffered reaction mixtures in which the pH of all components had been adjusted to 6.2 before mixing. Negligible changes in pH occurred during enzyme assays.

A variety of chemical inhibitors were tested for their effects on the IAAld oxidase, and the results are shown in Table II. N-ethylmaleimide, iodoacetate, and $HgCl_2$ inhibited this enzyme, suggesting a requirement for a free sulfhydryl group for full activity (13). IAAld oxidase was also strongly inhibited by EDTA, azide, and fluoride. These reagents are known (14) to be reactive toward certain divalent metal cations (Mg^{2+}, Mn^{2+}, Ca^{2+}), and we take the results as possibly indicative of a divalent metal cation requirement. This interpretation was supported by our observation of enhanced IAAld oxidase yield upon inclusion of 1 mM $MgCl_2$ in the original homogenization medium.

The inhibition of cucumber IAAld oxidase by 2,4-dichlorophenol and *p*-nitrophenol is noteworthy in light of the known inhibition of flavin-linked oxidases by substituted phenols (16). This IAAld oxidase activity is not dependent upon added pyridine nucleotide or FAD, and the analogy of this reaction with those catalyzed by other flavin-linked aldehyde oxidases (4) allows the suggestion that this IAAld oxidase may depend upon a flavin prosthetic group. The actual mechanism of action of this enzyme can only be determined when a homogeneous preparation is available.

Substrate Specificity. A direct substrate specificity study of the IAAld oxidase is difficult in the absence of an assay which is independent of the nature of the aldehyde substrate. The Salkowski assay employed throughout these experiments is specific for the IAAld/IAA couple. However, it is possible to

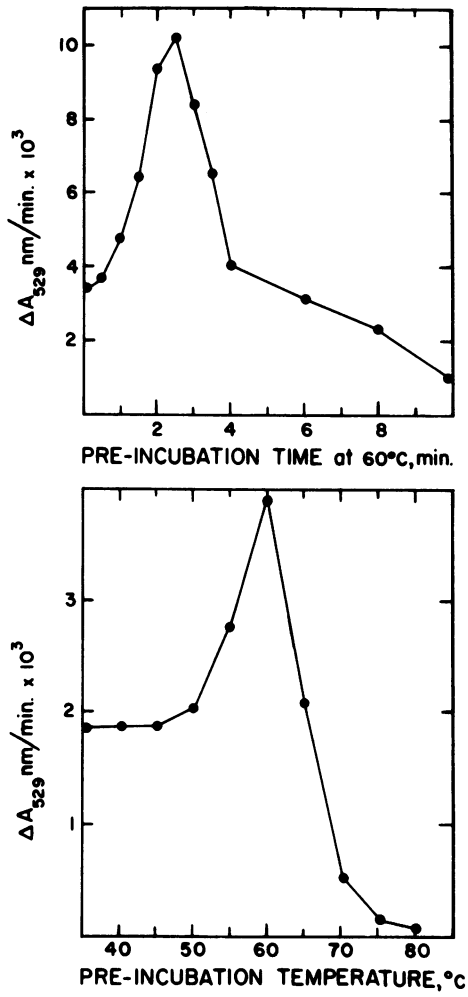

FIG. 1. Activation of cucumber IAAld oxidase by heat. Crude enzyme preparations were placed in a water bath at the temperature indicated, plunged into ice water at the appropriate time, and assayed at room temperature. Top: effect of length of heating at 60 C; bottom: effects of 2.5-min exposures to various temperatures.

TABLE I. *Identification of product of action of IAAld oxidase on indoleacetaldehyde*

Aliquots of reaction mixtures subjected to thin layer chromatography in 3 solvent systems. Solvent I: *iso*propanol - NH_4OH - water (10:1:1, v/v); Solvent II: diethyl ether - hexane (7:1, v/v); Solvent III: ethyl acetate - NH_4OH - *iso*propanol (9:7:4, v/v).

Sample	R_F Solvent I			Solvent II			Solvent III		
Indole-3-ethanol	0.88	--	--	0.46	--	--	0.93	--	--
Indole-3-acetaldehyde	--	0.90	--	--	0.71	--	--	0.97	--
Indole-3-acetic Acid	--	--	0.45	--	--	0.40	--	--	0.33
IAAld + Boiled Enzyme	--	0.91	--	--	0.71	--	--	0.97	--
IAAld + Enzyme	--	0.92	0.46	--	0.72	0.38	--	0.97	0.33

obtain a qualitative estimate of the specificity of the active site by noting the degree of inhibition of IAAld oxidation in the presence of other potential substrates. In this study, equal concentrations of IAAld and another aldehyde were incubated with the IAAld oxidase preparation and the rates of IAA production compared with the rate observed with IAAld alone. Results are shown in Table III.

It can be seen that, under these conditions, short chain aliphatic aldehydes interfered very little with IAAld oxidation by this enzyme preparation and that the efficacy of this slight inhibition decreased as the length of the alternate substrate increased from 2 to 4 carbons. It is unlikely that the activity of the short chain aldehydes was underestimated owing to evaporation from the reaction mixtures, since the reaction time was only 10 min. Certain aromatic aldehydes inhibited the IAAld oxidase rather strongly, with benzaldehyde being consistently more effective than either phenylacetaldehyde or indole-3-aldehyde. It is interesting that *trans*-cinnamaldehyde was a poor inhibitor under these conditions. This indicates a degree of structural specificity required of aromatic aldehydes, which is violated by the 3-carbon, double-bonded aldehyde side chain of this compound.

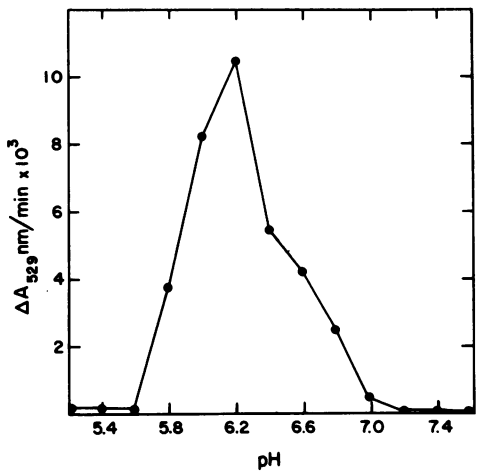

FIG. 2. Effect of pH on activity of cucumber IAAld oxidase. Aliquots of enzyme preparation were adjusted to pH 5 and centrifuged to remove precipitated protein. Following adjustment of pH to the indicated values, the aliquots were assayed in unbuffered reaction mixtures at the same pH values.

TABLE II. *Effects of inhibitors on cucumber indoleacetaldehyde oxidase*

Data of 2 experiments (2 enzyme preparations) are presented. Results expressed as per cent inhibition of water controls.

Inhibitor	Concentration	Inhibition		
		Expt. 1	Expt. 2	Average
	mM	% of control		
EDTA	10	89	92	91
Sodium Azide	1	22	17	20
	10	64	45	55
Sodium Fluoride	1	3	10	7
	10	43	52	48
Mercuric Chloride	0.01	100	100	100
Iodoacetate	1	6	15	11
	10	100	100	100
2,4-Dinitrophenol	0.1	5	1	3
	1	5	2	4
2,4-Dichlorophenol	0.1	14	20	17
	1	33	35	34
p-Nitrophenol	1	70	75	73
N-Ethylmaleimide	1	33	20	27
	10	64	50	57

TABLE III. *Inhibitory effects of potential alternate substrates on oxidation of indoleacetaldehyde by IAAld oxidase*

Various aldehydes were added to the reaction mixture at a concentration identical to that of IAAld. In Expt. 1, IAAld and competing aldehydes were present at 0.20 mM; in Expt. 2, 0.28 mM. Results are given as per cent inhibition of the rate with water in place of the alternate aldehyde.

Alternate Substrate	Inhibition	
	Expt. 1	Expt. 2
	% of control	
Acetaldehyde	12	10
Propionaldehyde	7	8
Butyraldehyde	5	2
trans-Cinnamaldehyde	7	5
Indole-3-aldehyde	--	25
Benzaldehyde	31	46
Phenylacetaldehyde	14	21

Inhibition by Auxin Analogs. The ability of reaction products (and their analogs) to inhibit the cucumber IAAld oxidase was explored in the concentration range from 0 to 0.66 mM. The auxin analog 2,4-D strongly inhibited the enzyme. At 0.66 mM 2,4-D, IAAld oxidase was inhibited by 25%, 53%, and 90% in three separate experiments with separately prepared enzyme. Inhibition by 2,4-D was observed in both untreated and heat-activated enzyme preparations. The presumed inhibitory effect of added IAA could not be tested directly under these assay conditions, since the presence of added IAA would have caused excessive Salkowski color development.

The results of the substrate specificity study previously discussed suggested that benzaldehyde and phenylacetaldehyde are also substrates for this enzyme, albeit less effective than IAAld. The effects of their presumed products, benzoic acid and phenylacetic acid, were tested. At concentrations up to 0.66 mM, benzoic acid produced no inhibitory effects, while phenylacetic acid led to a 10 to 20% inhibition at 0.6 mM. Neither these compounds nor 2,4-D interfered with the Salkowski reaction itself. We may conclude that 2,4-D and phenylacetic acid (and, by structural analogy, IAA) are inhibitors of cucumber IAAld oxidase and that this property is not well shared by the molecular analog benzoic acid, which lacks auxin activity. Resolution of the mechanism of this auxin-directed inhibition must await further purification and stabilization of the enzyme, but it is interesting to speculate that this property may play a regulatory role in auxin synthesis. It is possible that the activation of cucumber IAAld oxidase by heat or mercaptoethanol treatment may reflect a regulatory process active through other agents in the living plant. (It is also possible that the effects of heat and mercaptoethanol may be to release the enzyme from an initially particulate form.)

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